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PRAC2: A New Gene Expressed in Human Prostate and Prostate Cancer

Pär Olsson, Akira Motegi, Tapan K. Bera, Byungkook Lee, and Ira Pastan*

Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

BACKGROUND. The database of human Expressed Sequence Tags was previously used to identify *PRAC* (Prostate 47:125–131, 2001), a novel gene specifically expressed in human prostate, prostate cancer, rectum, and distal colon. In this report, we have identified *PRAC2*, another gene with a similar expression pattern that is located adjacent to the original *PRAC* gene on chromosome 17q21.3.

METHODS. Using a computer-based analysis, a cluster of sequence homologous ESTs was identified that is mainly derived from human prostate cDNA libraries. The tissue specificity was examined by multiple tissue RNA dot blots and RT-PCR. The *PRAC2* transcript and protein were identified using Northern blot analysis, RACE-PCR, primer extension, and Western blots.

RESULTS. *PRAC2* encodes a 564 nucleotide RNA found in prostate, rectum, distal colon, and testis. Weak expression was also found in placenta, peripheral blood leukocytes, skin, and in two prostate cancer cell lines: LNCaP and PC-3. The transcript seems to encode a 10.5-kDa nuclear protein. The *PRAC2* gene is located on chromosome 17 at position 17q21, between the *Hoxb-13* gene and the recently discovered *PRAC* gene.

CONCLUSIONS. Because of the higher expression of PRAC2 in prostate and its proximity to *Hoxb-13*, PRAC2 may have a function in prostate growth and development. *Prostate 56: 123–130, 2003.* © 2003 Wiley-Liss, Inc.

KEY WORDS: **EST; LNCaP; *Hoxb-13*; CLL**

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer and one of the leading causes of cancer death in males in the United States [1]. For prostate cancer that has metastasized, there are currently no curative therapies available. Studies to control prostate cancer have focused on early detection of prostatic carcinoma and developing new targets for therapy. Both the detection and treatment of cancer would potentially benefit from the identification of new genes that are specifically expressed in prostate cancer. The database of Expressed Sequence Tags (dbEST) contains sequences that originate from cDNA libraries, each of which is prepared from particular tumors, organs, or cell types. Therefore, analysis of ESTs provides valuable information about the expression of a particular gene in the corresponding tissues. We have previously shown that the dbEST is a useful tool to identify prostate-specific genes [2]. Several genes with a specific or differential expression in prostate have recently been published by our group [3–7]. One of the recent discoveries was the *PRAC* gene (prostate/rectum and colon), which codes for a 6-kDa protein [8]. PRAC is a nuclear protein

Abbreviations: CLL, chronic lymphocytic leukemia; dbEST, database of expressed sequence tag; FBS, fetal bovine serum; HEK, human embryonic kidney; LOH, loss of heterozygosity; PCR, polymerase chain reaction; PRAC2, Prostate/Rectum And Colon protein no. 2; RACE, rapid amplification of cDNA ends.

Pär Olsson and Akira Motegi have contributed equally to this study.

Pär Olsson's present address is Ludwig Institute for Cancer Research, 605 Third Avenue, New York, NY 10158.

*Correspondence to: Ira Pastan, Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, 37 Convent Drive, Room 5106, MSC 4264, Bethesda, MD 20892-4264. E-mail: pastani@pop.nci.nih.gov

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expressed in prostate, rectum, and distal colon. During the investigation of *PRAC*, we found another small gene on chromosome 17, situated between *PRAC* and the homeodomain *Hoxb-13* gene. This is of particular interest because the genes have a central role in the regulation of tissue differentiation. EST cluster data indicates that this new gene has a similar expression pattern to *PRAC*. In this report, we describe the characterization of this new gene, named *PRAC2*.

MATERIALS AND METHODS

Primers

Primers used in this study are listed as follows: C77RG2:1, 5′-CCA CAC CAC TAA TTA TTA TGG CGA G-3′; C77RG2:2, 5′-CCC TTC CGA CAG AGG CTC AC-3′; C77RG2:3, 5′-CGA AAC CTT CTT TTG CCC CC-3′; C77RG2:4, 5′-CTC GTG GGT ACT GAG CTG CGT GTG-3′; C77RG2:5, 5′-TGC AAG TTT ACG GTT CCA TAC AAG T-3′; C77RG2:6, 5′-TCG GCA GAT GTA TTG GTC CAG-3′; RG2:pext1, 5′- TAA ACT TGC AAT AAA GCC CGG-3′; RG2:pext2, 5′-GGG CAA AAG AAG GTT TCG TT-3′; RG2:pext3, 5′-CAC ACA CTT TTT TTC GGA TTT ACT TG-3′; RG2:Inv1, 5′-AAG TGA ATT CGA ATC TTA GAG ATT TAT TCC ACT GGT GT-3′; RG2:Inv2, 5′-AAG TCT CGA GCT CCC GAG CTT TCT CCC CTC TT-3′; RG2:Inv3, 5′-AAG TCT CGA GCT TCC CTG GAG AGC GAC TGT TCG G-3′; 77-1, 5′-TGC AAA CAG AGC GCC ACT G-3′; 77-2, 5′-GAA ATT CCA GAA TTA CAG GCT GAG-3′. All primers were synthesized by Lofstrand Labs Ltd. (Gaithersburg, MD).

Dot Blot and Northern Blot Hybridizations

Hybridizations on a human multiple tissue mRNA dot blot (RNA Master Blot, Clontech, Palo Alto, CA), multiple tissue Northern blot (MTN, Clontech), and on a matched tumor/normal expression array (Clontech) were carried out as previously described [5]. The probe used was the PCR fragment from primers C77RG2:1 and C77RG2:2 labeled with ^{32}P by random primer extension (Lofstrand Labs Ltd.).

PCR and 5′-RACE-PCR

PCR for rapid scan was carried out by using first strand cDNA from a Rapid-Scan Panel (Origene Technologies, Rockville, MD) and primers C77RG2:1 and C77RG2:2 according to the instructions. *PRAC2* mRNA expression was also examined on two prostate carcinoma cell lines, LNCaP and PC-3. Cell line mRNA was isolated by FastTrack mRNA isolation kit (Invitrogen, Carlsbad, CA). cDNA was prepared by reverse transcription using MMLV (Life Technologies, Gaithersburg, MD) with oligo-dT priming and PCR was performed with primers 77-1, 77-2 (PRAC), C77RG2:1, and C77RF2:2 (PRAC2). RACE-PCR was conducted using LNCaP mRNA or prostate tumor total RNA and primers RG2:pext1 and RG2:pext2 by following the instructions provided by the SMART-RACE PCR kit (Clontech). Several individual clones from the RACE product were isolated and sequenced to establish the correct *PRAC2* sequence.

Primer-Extension Analysis of RNA

The transcription start site of the *PRAC2* gene was determined by primer extension as described previously [9]. Briefly, 5 μg of LNCaP mRNA were mixed with 0.08 pmol of ^{32}P end-labeled RG2:pext1 primer. The mixture was ethanol precipitated and dissolved in 5× first strand buffer (Life Technologies). After 2 min of heating at 75°C and 30 min of incubation at 60°C, a primer-extension mix containing actinomycin D, dNTPs, DTT, and 20 U of MMLV-reverse transcriptase (Life Technologies) was added. The reaction was incubated for 1 hr at 37°C and terminated by addition of ethanol. The sample was resuspended in formamide-containing loading buffer (Promega, Madison, WI) and subsequently electrophoresed on a 6% polyacrylamide-urea gel. The gel was dried on Whatman paper and subjected to autoradiography.

Constructs

Construction of mammalian expression vectors for PRAC2/ORFs tagged with myc–His epitopes was performed as follows. The cDNA fragment for each open reading frame (ORF) was obtained by PCR using full-length PRAC2 cDNA as template with primers; 5′-ATG AAT TCA TTA TTA TGG CGA GGA AGA TAA AGA AGA C-3′ (ORF1, sense); 5′-ACC TCG AGC CAG GCT TCC AGC GGC CTG GG-3′ (ORF1, antisense); 5′-ATG AAT TCG AAG ACA TGG ACA GAA GGC GG-3′ (ORF2, sense); 5′-ACC TCG AGC CCA CTC CAG GAG AAC CTG CG-3′ (ORF2, antisense); 5′-ATG AAT TCG AGA TGG CTC GTA CCG AAC-3′ (ORF3, sense); 5′-ACC TCG AGC AAA AGA CGT GGG CGC CCC CTT C-3′ (ORF3, antisense). The obtained fragments were introduced into pcDNA3.1/myc–His vector version C (Invitrogen). All constructs were verified by DNA sequencing.

Cell Culture

LNCaP and PC3 cells were maintained in RPMI-1640 medium (Quality Biological, Inc., Gaithersburg, MD) at 37°C with 5% CO_2. The medium contained 10% fetal bovine serum (FBS, Quality Biological, Inc.), 2 mM

L-glutamine, 1 mM sodium pyruvate and penicillin/streptomycin. Human embryonic kidney (HEK) 293T and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium with 10% FBS, 2 mM glutamine and penicillin/streptomycin.

Transient Transfections and Subcellular Fractionation

HEK 293T cells were transiently transfected with plasmids expressing each ORF of PRAC2 tagged with myc–His epitope using calcium phosphate method. The preparation of total cell lysate was essentially the same as described previously [8]. For subcellular fractionation, 1×10^6 NIH3T3 cells were transiently transfected with PRAC2/ORF2/myc–His using FuGene (Roche). Fractionation was performed as described [8]. Briefly, cells were washed, harvested in PBS, and allowed to swell on ice for 15 min in buffer A (10 mM HEPES, pH 7.9/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM DTT/1 mM PMSF/1 μg/ml aprotinin/1 μg/ml leupeptin). Cells were disrupted by using a Dounce homogenizer and pestle B (20 strokes). Nuclei were pelleted by centrifuging at 1 k × g for 10 min. The supernatant was further separated by centrifuging at 100 k × g for 1 hr to obtain cytoplasmic (supernatant) and membrane (pellet) fractions. The nuclear pellet was washed with buffer B plus 0.1% Triton X-100 to remove residual membranes and resuspended in 1× sample buffer (62.5 mM Tris 6.8/1% SDS/10% glycerol) with brief sonication to shear DNA. The membrane pellet was resuspended in sample buffer with 1% Triton X-100.

Western Blot Analysis

Twenty μg of protein from extracts of transiently transfected cells or 10 μg protein from subcellular fractions were separated on a 16.5% Tris Tricine gel (Bio-Rad) and then transferred to a PVDF membrane (Bio-Rad). The membrane was probed with anti-myc antibodies (9E10, Santa Cruz) and detected using a chemiluminescence Western blotting kit according to the manufacturer's instructions (Amersham-Pharmacia Biotech).

Immunofluorescence

NIH 3T3 cells were grown on cover slips and transiently transfected with the PRAC2/ORF2/myc–His expression vector using FuGene (Roche). Thirty hr after transfection, the cells were fixed with 3.7% formaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 2% normal goat globulin/10% saponin in PBS. Staining was performed by incubating cells with anti-myc antibodies (9E10, 2 μg/ml) followed by TRITC-conjugated anti-mouse IgG (10 μg/ml, Molecular Probes) as a secondary antibody and 4′,6′-diamino-2-phenylindole (DAPI). After washing with PBS, the cells were examined and photographed by confocal laser microscopy (Zeiss LSM 510).

RESULTS

Restricted Expression of *PRAC2*

As part of our search for prostate-specific genes, we recently identified a cluster of ESTs that led to the discovery of the *PRAC* (Prostate, Rectum And distal Colon) gene [8]. A database analysis of the genomic region surrounding the *PRAC* gene reveals another EST cluster, which also contains ESTs that are mainly derived from prostate cDNA libraries (Fig. 1). This cluster contains 15 ESTs from normal prostate libraries (Pr22, Pr28), and three ESTs from other organs; one colon tumor EST, one chronic lymphocytic leukemia (CLL) EST, and one Ewing's sarcoma EST. Because the EST cluster analysis indicated a similar expression pattern as for the *PRAC* gene, this new cluster was named *PRAC2*.

By assembling the 18 ESTs, we obtained a 493-nucleotide sequence that contains a polyadenylation site (AATAAA). The clustering also indicates that the *PRAC2* gene contains two exons, one short 5′ exon and one longer 3′ exon (Fig. 1). The exon/intron junctions conform to the general GT-AG rule [10]. As with the *PRAC* cluster, the *PRAC2* cluster suggests that the *PRAC2* transcript is differentially expressed in prostate tissues. The gene is transcribed in the opposite direction of the *PRAC* gene and is located between the *Hoxb-13* gene and the *PRAC* gene.

To investigate tissue expression, we performed a PCR analysis using cDNAs from 24 different tissues. As shown in Figure 2A, the expected 332 bp PCR product was evident in prostate when primers C77RG2:1 and C77RG2:2 were used (lane 18). However, this 332 bp product was also detected in placenta, testis, muscle, colon, PBL, and skin. The signal in prostate was the strongest with testis being the second strongest. A different size band was present in heart; sequencing showed this band is a PCR artifact. *PRAC2* could also be detected by RT-PCR in the prostate cancer cell lines LNCaP and PC3 (Fig. 2B).

To confirm the PCR expression data (shown in Fig. 2A), we conducted a RNA dot blot analysis. Among the 61 different samples of normal adult and fetal tissues, *PRAC2* was detected in prostate (E8) and in some regions of the large bowel (B6 and C6). A faint signal could be detected in testis (F8) (Fig. 2C). In contrast to *PRAC*, where an equally strong signal could be detected in the rectum as in prostate [8], the prostate

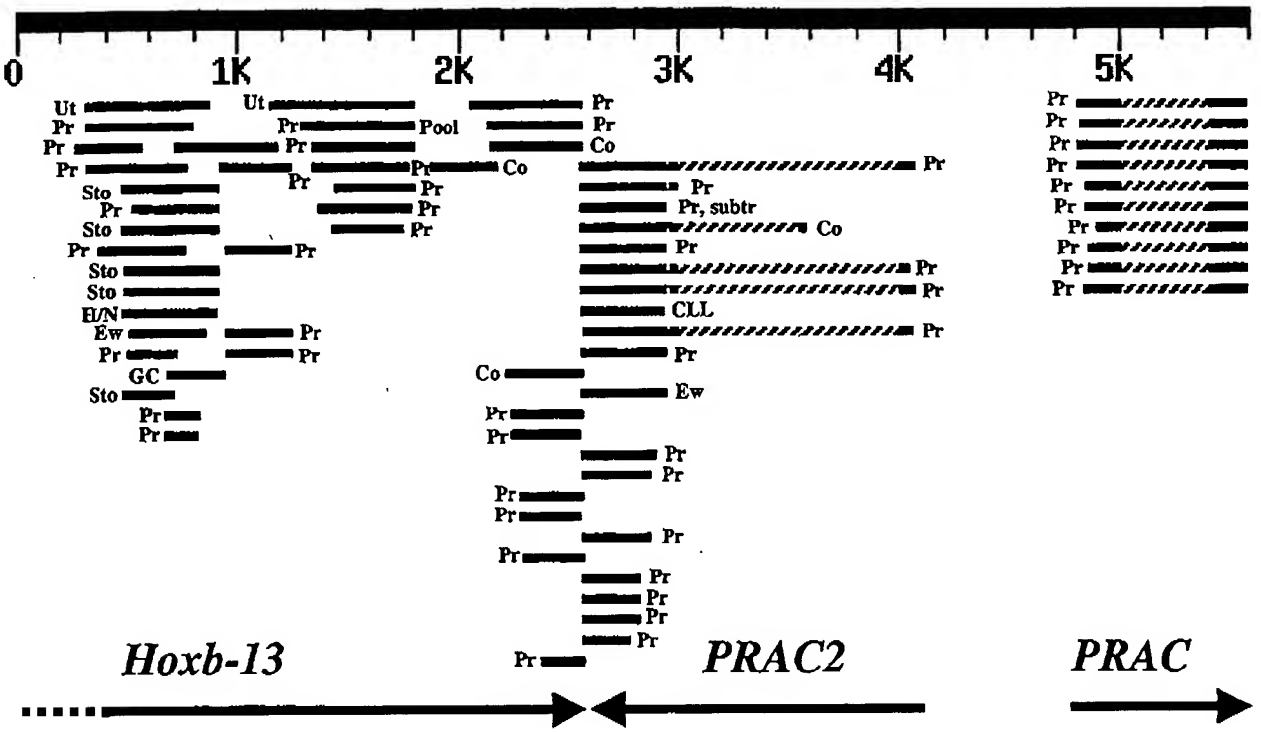

Fig. 1. The *PRAC* and *Hoxb-13* genes located on chromosome 17q21.3. The genes direction and location are schematically shown with arrows. The novel *PRAC2* gene is located between the recently discovered *PRAC* [8] gene and the previously known *Hoxb-13* gene but transcribed in the opposite direction. Schematically shown are the human ESTs that map to this genomic region. The abbreviations for the origins of the ESTs are: Pr, Prostate (normal or cancer); Co, colon; Ew, Ewing's sarcoma; CLL, chronic lymphocytic leukemia; Pool, pooled EST library from many tissues; Ut, uterus, Sto, stomach; and H/N, head and neck tumor. The *PRAC2* prostate ESTs are all from normal prostate libraries. Only the 3′ end of the *Hoxb-13* gene is shown.

gave a stronger signal for *PRAC2*. These data indicate that *PRAC2*, like *PRAC*, is restricted in its expression to a few tissues and is predominant in the prostate, rectum, and testis. Because of the similar expression pattern as PRAC, we named the new gene *PRAC2* (prostate/rectum and colon number 2).

To evaluate the size of the *PRAC2* transcript, Northern blot hybridizations were performed. A major band of about 500–600 bp was detected in prostate (Fig. 3A).

Full-Length PRAC2 Transcript

To determine if the *PRAC2* sequence assembled from the EST sequences was full-length, a primer extension experiment was carried out to obtain the transcription start site. A single band of approximately 135 bp was obtained using primer RG2:pext1 and mRNA from LNCaP cells (data not shown). We calculated that there were 70–72 base pairs missing from the 5′end of the *PRAC2* transcript in the EST contig sequence.

To obtain a full-length *PRAC2* cDNA clone, 5′-RACE-PCR was conducted using LNCaP mRNA and primers RG2:pext1 and RG2:pext2. Several clones isolated by 5′-RACE-PCR was sequenced. An extra 71 bp were obtained by 5′-RACE-PCR, which is consistent with the transcription start site as determined by primer extension. The full-length cDNA contains a total of 564 bp excluding the poly(A) tail (Fig. 3B).

Examination of the DNA sequence indicates that there are three possible ORFs in the *PRAC2* transcript. The first ATG, at position 195, predicts a 82 amino acid protein with a calculated molecular mass of 8.9 kDa. The second ATG, at position 218, is in a different reading frame. It encodes a protein of 90 amino acids, with a calculated molecular mass of 10.5 kDa. The third possible ORF starts at position 286, and it encodes a protein of 87 amino acids and a molecular mass of 9.0 kDa. None of the ORFs span the exon 1 and 2 boundary at position 134 (Fig. 3B). The nucleotide sequence of full-length *PRAC2* cDNA has been deposited in GenBank database with accession no. AF532777.

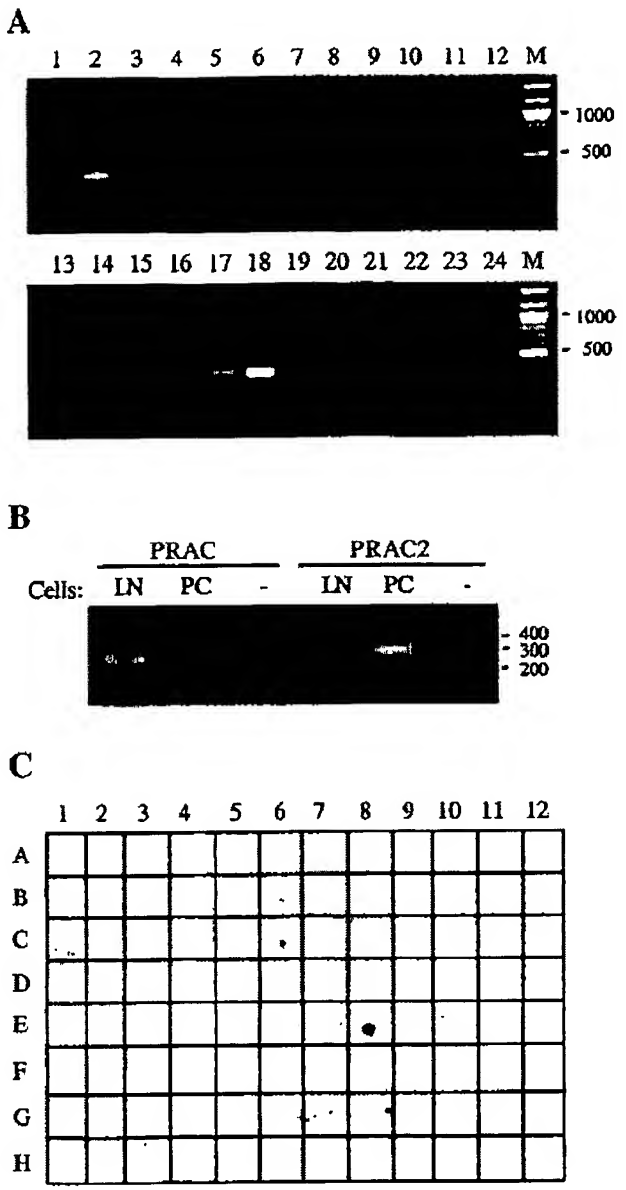

Fig. 2. Prostate specificity of the *PRAC2* cluster. **A**: PCR on cDNA from 24 different human tissues showed strong expression in prostate (**lane 18**) and moderate to weak expression in placenta (**lane 1**), testis (**lane 2**), muscle (**lane 4**), colon (**lane 7**), peripheral blood leukocytes (**lane 16**), and skin (**lane 17**). The expected size of the PCR product is 332 bp. The lower size band in heart (**lane 11**) is an artifact. **B**: RT-PCR analysis in prostate cell line LNCaP (LN) and PC-3 (PC). The *PRAC2* gene transcript, as well as that of *PRAC* gene, is present in both cell lines. **C**: Hybridization of a *PRAC2* probe (the same region that was PCR amplified in A) to an RNA dot blot containing mRNA from 61 normal human cell types or tissues. Prostate (E8), and different regions of the large bowel are weakly positive; rectum (C6), transverse colon (A6), and descending colon (B6). A weak signal could also be detected in the colon carcinoma cell line SW480 (G10). Positive controls (G12 and H12, human DNA) also show a weak signal.

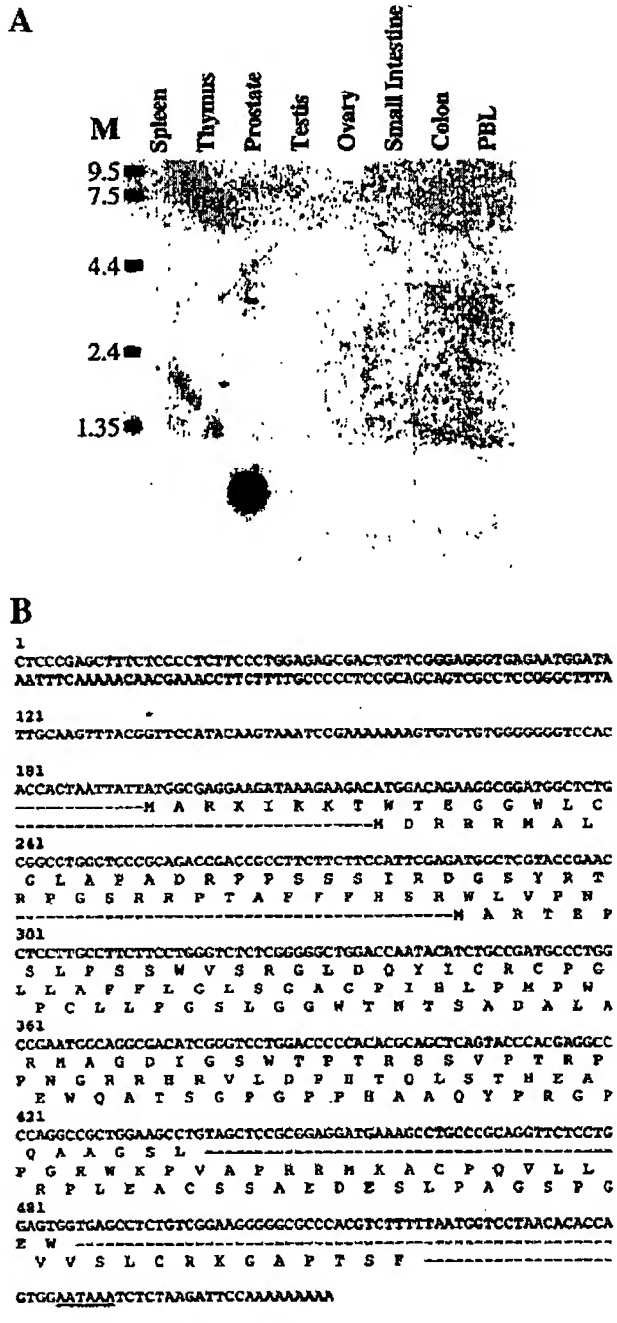

Fig. 3. The *PRAC2* transcript. **A**: A Northern blot experiment using the same *PRAC2* probe as in the Dot Blot experiment (see Fig. 2). **Lane M** is the molecular weight marker in kb. **B**: DNA sequence of the full-length *PRAC2* transcript. Shown also are the sequences for the three possible ORFs. The splice site at position 134 is indicated with an asterisk. The polyadenylation site at position 525 is underlined.

PRAC2 Transcript Encodes a Small Nuclear Protein

Often the ORF, which is most efficiently translated in vitro, is also used by translation mechanisms in vivo. To identify which of the three ORFs encodes the PRAC2 protein, we transiently transfected 293T cells with cDNAs encoding three different reading frames tagged with myc–His epitopes. Forty-eight hours after transfection, the cells were lysed and 20 μg of total cell extract was separated on a 16.5% SDS–PAGE gel followed by western immunoblot analysis. An abundant 14 kDa band was present in the ORF2 lane suggesting that the second ORF (ORF2) is the reading frame that is strongly translated (Fig. 4A). A very weak 12-kDa band was generated from ORF1.

We next carried out cellular fractionation experiments to determine the cellular location of the PRAC2 protein. NIH3T3 cells were transiently transfected with a myc–His tagged ORF2. Nuclear, cytosolic and membrane fractions were separated by centrifugation and analyzed by Western blots using anti-myc epitope antibodies. PRAC2 was mainly detected in the nuclear fraction with a minor amount found in the membrane fraction. No signal was detected in the 100,000 × g supernatant (Fig. 4B). To confirm and extend the biochemical localization experiments, we performed immunofluorescence studies. Immuno-staining of NIH 3T3 cells expressing myc–His tagged ORF2 with anti-myc antibodies showed that more than half of positive cells have intense nuclear staining as well as a granular pattern within the cytoplasmic area. The rest of the positive cells showed only cytoplasmic granular signals devoid of nuclear staining (Fig. 5). No signal was detected in ORF2-expressing cells treated with the secondary antibody alone (data not shown). These data indicate that PRAC2 is located in the nucleus but is also found outside the nucleus. Whether these extra-nuclear structures are aggregates of protein in the cytosol or represent PRAC2 associated with some type of membranes is under investigation.

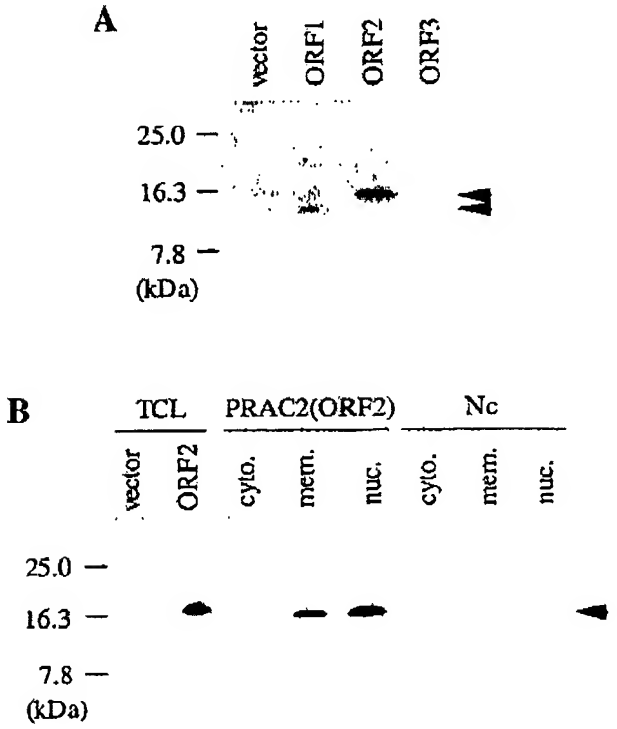

Fig. 4. Transient expression of the three possible ORFs in mammalian cells. **A**: HEK 293T, cells were transiently transfected with myc–His plasmids expressing myc–His tagged three possible ORFs of PRAC2 (ORF1-3). The second ORF seems to be the one that is predominantly expressed. **B**: Subcellular fractionation of NIH3T3 cells untransfected (Nc) and transiently transfected with ORF2 of PRAC2. PRAC2 localized to the nucleus. Shown are also total cell lysates (TCL) of untransfected (Nc) and transfected cells (ORF2).

DISCUSSION

Using the EST database as a guide, we have identified the *PRAC2* gene that lies between the *Hoxb-13* and *PRAC* genes. We found that *PRAC2* RNA is expressed at high levels in normal prostate and testis. Lower expression was found in placenta, muscle, colon, PBL, and skin. Expression was also found in the prostate cell lines LNCaP and PC-3.

The absence in the EST database of *PRAC2* ESTs from several of the tissues that were positive in the RT-PCR and dot blot experiments is presumably due to the incomplete dbEST. It is clear that the expression pattern for *PRAC2* is broader than for *PRAC*, although both are predominantly expressed in prostate and colon. The two genes lie close together but are transcribed in opposite directions. It is possible that they use the same regulatory elements to govern their pattern of expression (Fig. 1). However, examination of the genomic region between the two RNA transcripts did not reveal characteristic promoter elements or androgen response elements that could explain coexpression in certain tissues.

The coding sequence of the *PRAC2* transcript has three possible ORFs. Initial attempts to generate specific antibodies against each ORF to detect the protein in tissue samples failed. Instead, transfection with DNAs encoding myc–His tagged proteins for all three ORF was performed, and these studies indicate that it is the second ORF that is expressed. This result shows that *PRAC2*, like *PRAC*, skips the first AUG, and the actual translation start site is 218 nucleotides downstream of the transcription initiation site. It has been suggested that the transcript, in which the first AUG is often

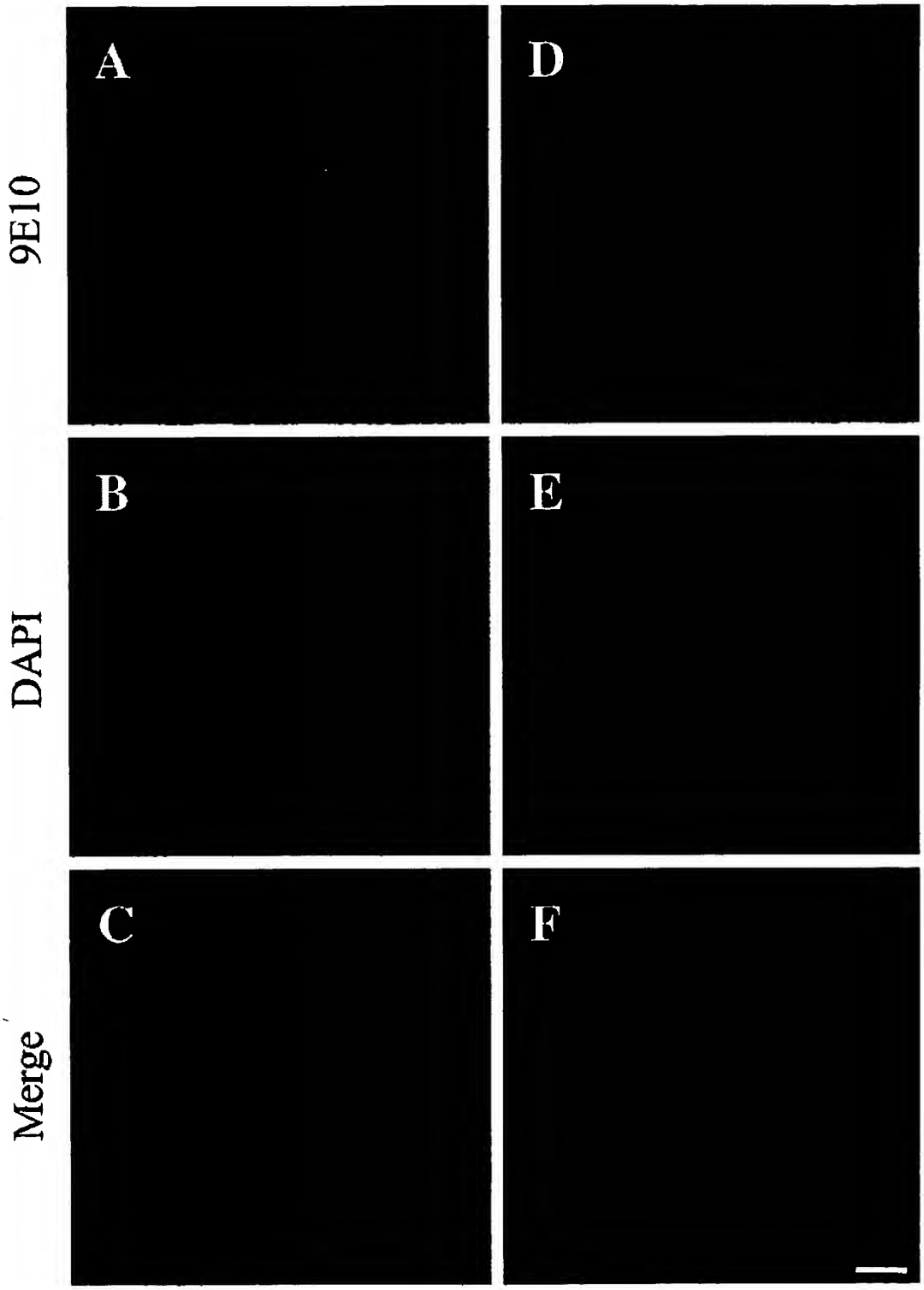

Fig. 5. Cellular localization of PRAC2 (ORF2) protein. NIH 3T3 cells were transfected with the myc–His tagged PRAC2 (ORF2) expression vector and immunostained with anti-myc antibody 9E10 (**A**, **D**) or stained with DAPI to visualize nuclei (**B**, **E**). **Panels C** and **F** show the merged images. Bar = 50 μm.

skipped, encodes regulatory proteins such as oncogenes, transcription factors, and proteins involved in the regulation of immune responses and in signal transduction pathways [11,12]. Given the indications that PRAC2 is mainly a nuclear protein, it might have some function in transcriptional regulation, which also has been suggested for PRAC.

The chromosomal location of *PRAC2* is 17q21.3, and directly downstream of the homeodomain *Hoxb-13* gene, which has been reported as sequence specific transcription factor, which is part of a developmental regulatory system that provides cells with specific positional identities on the anterior–posterior axis [13]. *PRAC2* and *Hoxb-13* are transcribed in the opposite directions and interestingly the polyadenylation signals for the respective genes are just 34 nucleotides apart (Fig. 1). It has been reported that in the adult mouse the *Hoxb-13* gene is expressed in prostate, rectum, and colon adjacent to rectum [14]. This expression pattern is similar to the pattern of expression we previously found for *PRAC*, and now *PRAC2*, in humans. Search of the human dbEST for expression of the *Hoxb-13* gene indicates a similar expression pattern in human prostate, colon, stomach, and uterus. This raises the possibility that *PRAC*, *PRAC2*, and *Hoxb-13* are under the same transcriptional control and may be involved in similar biological pathways.

Chromosome 17q21, where the *PRAC* genes are located, has been shown to undergo loss of heterozygosity (LOH) in prostate cancer [15,16]. However, the region with the highest LOH seems to be located close to, or within, the *BRCA1* gene, which is slightly closer to the centromere than the *PRAC* genes. The *PRAC* genes are located close to the *Hoxb-2* gene, which was reported to have a relatively low LOH, around 5% [17]. If the *PRAC* genes were located within the LOH region, there could possibly be a reduction in *PRAC*/*PRAC2* expression in prostate cancer. However, no such decrease could be found for PRAC [8]. Further studies are needed to understand the role of the *PRAC* genes in prostate cancer development.

PRAC and *PRAC2* both have high expression in prostate and both encode small nuclear proteins. Further investigations of the function of these genes should reveal what role they might play in prostate function.

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